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Protein for Inhibiting Apoptosis

The present invention relates to a protein which is suitable for inhibiting apoptosis, a DNA encoding said protein and a method of producing the protein. The invention also relates to the use of the DNA and the protein as well as to antibodies directed against the protein.

Apoptosis is programmed cell death. It is used e.g. by the immune system to keep off harmful substances, such as viruses. For this purpose, virus-specific T lymphocytes attack those cells of the body which are virus-infected, and kill them by releasing apoptosis-induced proteins such as perforin. The T lymphocytes can also express the CD95 (APO-1/Fas) ligand, so that cell death proceeds via the CD95 route. This route comprises the binding of the CD95 ligand to the CD95 receptor which then interacts with the adapter protein FADD, so that the recruitment and the activation of the protease FLICE are induced at the DISC („death-inducing signaling complex").

Further studies refer to the fact that apoptosis is also co-responsible for the development of various diseases. Such diseases are e.g. AIDS, autoimmune diseases and neurodegenerative diseases. In order to take steps against these diseases, it would be helpful to have substances which can inhibit apoptosis. However, such substances have been known only insufficiently by now.

Therefore, it is the object of the present invention to provide a product by which apoptosis can be inhibited.

According to the invention, this is achieved by the subject matters defined in the claims.

Thus, the subject matter of the present invention relates to a protein which is suitable for inhibiting apoptosis, the protein comprising the amino acid sequence of figure 1 or an amino acid sequence differing therefrom by one or several amino acids.

The present invention is based on the applicant's finding that in animals, particularly mammals, more particularly human beings, a protein exists which can inhibit apoptosis. This protein comprises the amino acid sequence of figure 1 or an amino acid sequence differing therefrom by one or several amino acids. Furthermore, the applicant found that the protein interacts with the adapter protein FADD, so that the recruitment and the activation of the protease FLICE are inhibited at DISC.

The above protein is referred to as FLIP („FLICE inhibitory protein") in the present invention.

A further subject matter of the present invention relates to a nucleic acid coding for FLIP. It can be an RNA or a DNA. The latter can be e.g. a genomic DNA or a cDNA. A DNA which comprises the following is preferred:

(a) the DNA of figure 1 or a DNA differing therefrom by one or several base pairs,
(b) a DNA hybridizing with the DNA from (a), or
(c) a DNA related to the DNA from (a) or (b) via the degenerated genetic code.

The expression „hybridizing DNA" refers to a DNA which hybridizes with a DNA from (a) under normal conditions, particularly at 20°C below the melting point of the DNA.

The DNA of figure 1 was deposited with the DSM (Deutsche Sammlung von Mikroorganismen und Zellkulturen [German-type collection of microorganisms and cell cultures]) as C-FLIP/2/W23795 and C-FLIP/1/AA115792 under DSM 11488 and DSM 11487, respectively, on March 25, 1997.

A DNA according to the invention is described below in the form of a cDNA. It is exemplary for every DNA falling under the present invention.

A cDNA according to the invention can be produced by common methods. It is favorable to use a human expression library as a basis and screen it with the DNA of figure 1, particularly with primers concerning the 5' region and 3' region, respectively, of the edged DNA region. Common, particularly the above, conditions can be chosen as hybridization conditions. Positive clones can then be tested for their apoptosis inhibitory activity by common methods.

A cDNA according to the invention can be present in a vector and expression vector, respectively. A person skilled in the art is familiar with examples thereof. In the case of an expression vector for E. coli these are e.g. pGEMEX, pUC derivatives, pGEX-2T, pET3b and pQE-8. For the expression in yeast, e.g. pY100 and Ycpad1 have to be mentioned while e.g. pKCR, pEFBOS, cDMB, pCEV4 and pEFrsFLAG have to be indicated for the expression in animal cells. The baculovirus expression vector pAcSGHisNT-A is particularly suitable for the expression in insect cells.

The person skilled in the art is familiar with suitable cells to express a cDNA according to the invention, which is present in an expression vector. Examples of such cells comprise the E. coli strains HB101, DH1, x1776, JM101,

JM109, BL21 and SG 13009, the yeast strain Saccharomyces cerevisiae and the animal cells L, 3T3, FM3A, CHO, COS, Vero, HeLa and BJAB as well as the insect cells sf9.

The person skilled in the art knows in which way a cDNA according to the invention has to be inserted in an expression vector. He is also familiar with the fact that this DNA can be inserted in combination with a DNA coding for another protein and peptide, respectively, so that the cDNA according to the invention can be expressed in the form of a fusion protein.

In addition, the person skilled in the art knows conditions of culturing transformed cells and transfected cells, respectively. He is also familiar with methods of isolating and purifying the protein expressed by the cDNA according to the invention. Thus, such a protein, which may also be a fusion protein, also represents a subject matter of the present invention.

A further subject matter of the present invention relates to an antibody directed against an above protein and fusion protein, respectively. Such an antibody can be prepared by common methods. It may be polyclonal and monoclonal, respectively. For its preparation it is favorable to immunize animals - particularly rabbits or chickens for a polyclonal antibody and mice for a monoclonal antibody - with an above (fusion) protein or a fragment thereof. Further „boosters" of the animals can be effected with the same (fusion) protein or with fragments thereof. The polyclonal antibody can then be obtained from the animal serum and egg yolk, respectively. For the preparation of the monoclonal antibody, animal spleen cells are fused with myeloma cells. An antibody according to the invention, which is directed against a protein having the amino acid sequence of figure 2, was deposited with the DSM as NF6 on April 1, 1998.

The present invention enables the detailed examination of apoptosis and its effect, particularly in the case of certain diseases, such as AIDS, autoimmune diseases and neurodegenerative diseases. By means of a nucleic acid according to the invention, particularly a DNA and primers derived therefrom, it can be identified whether mammals, particularly human beings, contain and/or express a gene which codes for a FLIP protein within the above meaning. For this purpose, the person skilled in the art will carry out common methods, such as reverse transcription, PCR reaction, hybridization and sequencing. A kit is also provided according to the invention, which contains an above nucleic acid, particularly DNA, and/or primers derived therefrom as well as carriers and conventional auxiliary agents.

Furthermore, the present invention is suited to inhibit apoptosis. This is of great significance in diseases, such as AIDS and neurodegenerative diseases. A FLIP protein according to the invention can be inserted in mammals, particularly human beings. For this purpose it can be favorable to couple FLIP to a protein which is not considered foreign by the particular body, e.g. transferrin or BSA. A nucleic acid according to the invention, particularly a DNA, can also be inserted in mammals, particularly human beings where it is expressed. For this purpose, it can be favorable to have the expression of the nucleic acid according to the invention controlled by a tissue-specific promoter. A person skilled in the art is familiar with vectors which are suitable for the expression of a nucleic acid in mammals. In addition, the expression of FLIP can be controlled and regulated with an antibody according to the invention. The antibody can also be present in the above kit.

Therefore, the present invention represents a great contribution to the diagnostic and therapeutic understanding of apoptotic processes. In this connection, the diagnostic survey cannot only be made postnatally but also already prenatally.

Brief description of the drawing:

Fig. 1 shows the base sequence and the amino acid sequence derived therefrom and comprised by a FLIP protein according to the invention. The edged sequence shows a DED (death effector domain) region. The sequence of figure 1 is found in DSM 11488.

Fig. 2 shows the base sequence and the amino acid sequence derived therefrom and comprised by a FLIP protein according to the invention. The sequence of figure 2 is found in DSM 11487.

Fig. 3 shows in (A) the expression of a FLIP protein according to the invention in cells. Because of the FLAG-Tag portion, the FLIP protein (FLIP-FLAG) according to the invention shows a slower running behavior than the FLIP protein expressed endogenously by the cells. In (B), the inhibitory effect of the FLIP protein according to the invention is shown on CD95-mediated apoptosis and in (C) its inhibitory effect is shown on the activation of the protease FLICE.

The present invention is explained by the below examples.

Example 1: Preparati n and purification f a FLIP protein according to th invention

For the preparation of a FLIP protein according to the invention the DNA of fig. 1 is provided with BamHI linkers, subsequently excised using BamHI and inserted in the expression vector pQE8 (Diagen company) cleaved by BamHI. The expression plasmid pQ/FLIP is obtained. Such a plasmid codes for a fusion protein comprising 6 histidine residues (N terminus partner) and the FLIP protein of fig. 1 according to the invention (C terminus partner). pQ/FLIP is used for transforming E. coli SG 13009 (cf. Gottesman, S. et al., J. Bacteriol. 148, (1981), 265-273). The bacteria are cultured in an LB broth with 10 µg/ml ampicillin and 25 µg/ml kanamycin and induced with 60 µM isopropyl-ß-D-thiogalactopyranoside (IPTG) for 4 h. Lysis of the bacteria is achieved by the addition of 6 M guanidine hydrochloride. Thereafter, chromatography (Ni-NTA resin) is carried out with the lysate in the presence of 8 M urea in accordance with the instructions from the manufacturer (Diagen company) of the chromatography material. The bound fusion protein is eluted in a buffer having a pH of 3.5. After its neutralization, the fusion protein is subjected to 18 % SDS polyacrylamide gel electrophoresis and stained with coomassie blue (cf. Thomas, J.O. and Kornberg, R.D., J. Mol. Biol. 149 (1975), 709-733).

It shows that a (fusion) protein according to the invention can be prepared in highly pure form.

Example 2: Preparation and detection of an antibody according to the invention

A fusion protein of Example 1 according to the invention is subjected to 18 % SDS polyacrylamide gel electrophoresis. After staining the gel with 4 M sodium acetate, an about 20-60 kD band is cut out of the gel and incubated in phosphate-buffered common salt solution. Gel pieces are sedimented before the protein concentration of the supernatant is determined by SDS polyacrylamide gel electrophoresis which is followed by coomassie blue staining. Animals are immunized with the gel-purified fusion protein as follows:

Immunization protocol for polyclonal antibodies in rabbits

35 µg of gel-purified fusion protein in 0.7 ml PBS and 0.7 ml of complete Freund's adjuvant and incomplete Freund's adjuvant, respectively, are used per immunization:

Day 0: 1st immunization (complete Freund's adjuvant)
Day 14: 2nd immunization (incomplete Freund's adjuvant; icFA)
Day 28: 3rd immunization (icFA)
Day 56: 4th immunization (icFA)
Day 80: bleeding to death.

The rabbit serum is tested in an immunoblot. For this purpose, a fusion protein of Example 1 according to the invention is subjected to SDS polyacrylamide gel electrophoresis and transferred to a nitrocellulose filter (cf. Khyse-Andersen, J., J. Biochem. Biophys. Meth. 10 (1984), 203-209). The Western blot analysis is carried out as described in Bock, C.-T. et al., Virus Genes 8, (1994), 215-229. For this purpose, the nitrocellulose filter is incubated with a first antibody at 37°C for one hour. This antibody is the rabbit serum (1:10000 in PBS). After several wash steps using PBS, the nitrocellulose filter is incubated with a second antibody. This antibody is an alkaline phosphatase-coupled monoclonal goat anti-rabbit IgG antibody (Dianova company) (1:5000) in PBS. 30 minutes of incubation at 37°C are followed by several wash steps using PBS and subsequently by the alkaline phosphatase detection reaction with developer solution (36 µM 5'-bromo-4-chloro-3-indolylphosphate, 400 µM nitro blue tetrazolium, 100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM MgCl2) at room temperature until bands are visible.

It shows that polyclonal antibodies according to the invention can be prepared.

Immunization protocol for polyclonal antibodies in chickens

40 µg of gel-purified fusion protein in 0.8 ml PBS and 0.8 ml of complete Freund's adjuvant and incomplete Freund's adjuvant, respectively, are used per immunization.

Day 0: 1st immunization (complete Freund's adjuvant)
Day 28: 2nd immunization (incomplete Freund's adjuvant; icFA)
Day 50: 3rd immunization (icFA)

Antibodies are extracted from egg yolk and tested in a Western blot. Polyclonal antibodies according to the invention are detected.

Immunizati n protocol for m n cl nal antibodies in mice

12 µg of gel-purified fusion protein in 0.25 ml PBS and 0.25 ml of complete Freund's adjuvant and incomplete Freund's adjuvant, respectively, are used per immunization. The fusion protein is dissolved in 0.5 ml (without adjuvant) in the 4th immunization.

Day 0: 1st immunization (complete Freund's adjuvant)
Day 28: 2nd immunization (incomplete Freund's adjuvant; icFA)
Day 56: 3rd immunization (icFA)
Day 84: 4th immunization (PBS)
Day 87: fusion.

Supernatants of hybridomas are tested in a Western blot. Monoclonal antibodies according to the invention are identified.

Example 3: Expression of a FLIP protein according to the invention in cells and its effect

(a) The DNA of figure 2 is provided with ExoR5-/XbaI linkers, subsequently excised using EcoRI and XbaI and inserted in the expression vector pEFrsFLAG cleaved by the same restriction enzymes. The expression plasmid pEFrsFLAG-FLIP is obtained. It codes for a fusion protein FLAG-FLIP from a FLAG-Tag (N terminus partner) and the FLIP protein of figure 2 according to the invention (C terminus partner). pEFrsFLAG-FLIP is used for the transfection of the human cells BJAB. Extracts are obtained from transfected cells and separated electrophoretically on an SDS polyacrylamide gel. Then, a Western blot method is carried out in which a monoclonal antibody of Example 2 is used for detecting the expressed FLIP protein. An anti-mouse antibody is used for the detection of the antibody binding (cf. figure 3A).

It shows that a FLIP protein according to the invention can be expressed in cells and detected by an antibody according to the invention.

(b) The above BJAB cells transfected with pEFrsFLAG-FLIP are incubated without treatment or with treatment with 10 ng/ml anti-APO-1 at 37°C for 16 hours. By the treatment using anti-APO-1, CD95-mediated apoptosis is induced. The amount of apoptotic cells is determined by identification of DNA fragmentation (cf. figure 3B).

It shows that CD95-mediated apoptosis can be inhibited by the expression of the FLIP protein according to the invention.

(c) BJAB cells and BJAB cells transfected with pEFrsFLAG-FLIP are treated with 1 µg/ml anti-APO-1. Cell extracts are collected and separated electrophoretically in an SDS polyacrylamide gel. Then, a Western blot method is carried out in which an antibody directed against the protease FLICE is used (cf. figure 3C).

It shows that in cells in which the FLIP protein according to the invention is expressed, the activation of FLICE, i.e. the cleavage into the active subunit p18, is prevented.

Claims

1. A protein suitable for inhibiting apoptosis, wherein the protein comprises the amino acid sequence of fig. 1 or an amino acid sequence differing therefrom by one or several amino acids.
2. The protein according to claim 1, comprising the amino acid sequence of fig. 2.
3. DNA coding for the protein according to claim 1, wherein the DNA comprises:
   (a) the DNA of fig. 1 or a DNA differing therefrom by one or several base pairs,
   (b) a DNA hybridizing with the DNA from (a), or
   (c) a DNA related to the DNA from (a) or (b) via the degenerated genetic code.
4. DNA according to claim 3, comprising the base sequence of figure 2.
5. Expression plasmid comprising the DNA according to claim 3 or 4.
6. Transformant containing the expression plasmid according to claim 5.
7. A method of producing the protein according to claim 1 or 2, comprising the culturing of the transformant according to claim 6 under suitable conditions.
8. Antibodies directed against the protein according to claim 1 or 2.
9. Use of the protein according to claim 1 or 2 as a reagent for inhibiting apoptosis.
10. Use of the DNA according to claim 3 or 4 as a reagent for diagnosis and/or inhibition of apoptosis.